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CXCL12 induces CaMKII synaptic localization and glutamate-induced hippocampal cell death

Robert M. Bragg III
Western Washington University

CXCL12 Induces CaMKII Synaptic Localization

And Glutamate-Induced Hippocampal Cell Death

By

Robert M. Bragg III

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Kathleen L. Kitto, Dean of the Graduate School

ADVISORY COMMITTEE

Chair, Dr. Jacqueline Rose

Dr. Jeffery Grimm

Dr. Michael Mana

MASTER'S THESIS

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Robert M. Bragg III

June 2014

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A Thesis Presented to

The Faculty of

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Abstract

Cerebral ischemia is known to induce cell death through hypoxia and oxidative stress following reperfusion. However, cell death can spread beyond the ischemic core when toxic glutamate levels act on NMDA receptors of surrounding regions. Levels of CXCL12, a chemokine primarily released from glia, reportedly elevate following ischemia. Acute activation of its receptor, CXCR4, appears to serve a neuroprotective function while prolonged activation results in cell death and this cell death was reported to be dependent on the combined release of calcium from intracellular stores as well as calcium influx through NMDARs. Calcium influx through NMDA receptor channels leads to activation of CaMKII, and it has also been shown that intracellular calcium can be sufficient to activate CaMKII. Thus, it is of interest to determine if cell death following CXCL12 application is mediated by CaMKII; and further, if exposure to CXCL12 primes neurons for glutamate-induced toxicity thus increasing cell death. Here, it was confirmed that acute (30 min) delivery of CXCL12 is not detrimental, while sustained activation (3 hour) resulted in a significant increase in cell death compared to controls. In addition, both acute and sustained CXCL12 exposure induced translocation of CaMKII to synapses. Delivery of a strong excitatory stimulus (500 µM glu + 10 µM gly) following either acute or sustained CXCL12 application appeared to elevate susceptibility to glutamate-induced cell death. Interestingly, pre-exposure to CXCL12 also induced cell death following delivery of a more physiologically relevant concentration of glutamate (100 µM glu + 10 µM gly). Peptide inhibitors that act to block CaM or ATP binding to CaMKII both appeared to reduce CXCL12-induced increases in cell death. These

results suggest that both acute and sustained CXCR4 activation increase neuron susceptibility to excitotoxicity and this priming is at least partially mediated by CaMKII.

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Chapter 1: Introduction

Introduction

Cerebral ischemic attack, commonly referred to as stroke, is the leading cause of disability, the second leading cause of dementia and the third leading cause of death worldwide (Bakhai, 2010). Due to this high prevalence, it is critical to determine the underlying mechanisms and causes of stroke-induced cell death. Interestingly, there is strong evidence that genetic factors influence the pathogenesis of stroke, but discovery of specific mutations has been limited (Hassan and Markus, 2000). Behavioral risk factors and preventative measures for stroke are fairly well defined; however, there is still a lack of awareness and a lack of emphasis of the behavioral factors which has led to the high stroke rates and problems associated with them (Gorelick, 2002). As clinicians continue to try and prevent stroke, researchers must continue to study the associated cellular mechanisms in order to discover new ways to mitigate brain damage once a stroke has occurred.

The primary objectives of this introduction are: (A) to summarize the pathophysiology of stroke, highlighting excitotoxicity and the inflammatory response; (B) discuss different sources of calcium entry and their implications for excitotoxicity; (C) highlight the role of CaMKII and its subsequent activity; (D) introduce chemokines; (E) discuss the potential link between CaMKII and chemokines; and (F) list the specific aims and outcomes.

Pathophysiology of stroke

At the gross level, strokes are generally caused by a vascular blockage or a hemorrhage which limits blood supply to a given brain area. This infarction translates to a loss of oxygen/glucose and results in a complex pathophysiological state; the ischemic core suffers from hypoxia, after which cell death is imminent—either by mechanisms of apoptosis or by necrosis (Deb et al., 2010). Necrotic cell death leads to release of cellular contents, including glutamate, which may be toxic to cells in the surrounding area (referred to as the penumbra) that were not directly affected by the ischemic event. In addition to excitotoxicity, the penumbra is subjected to potentially deleterious effects arising from ionic imbalances and activation of inflammatory pathways. However, cell death in the penumbra is considered secondary to the stroke itself and may be preventable through early interventions.

Excitotoxicity

The term "excitotoxicity" was first coined by Olney (1969), following the observation that over-excitation of neurons with glutamate results in their death. Since then, many of the underlying mechanisms have been elucidated. These include rises of intracellular calcium mediated by calcium permeable ion channels; specifically, NMDA receptors (Tymianski, Charlton & Carlen, 1993), and to a lesser extent L-type voltage gated calcium channels (L-VGCCs; Brewer et al., 2007; Stanika et al., 2012).

In normal excitatory conditions, increased intracellular calcium activates a multitude of pathways, notably the calcium/calmodulin (CaM)-dependent kinase II (CaMKII) signaling cascade which mediates learning and memory through mechanisms of LTP and LTD (Lisman et al., 2002). In hyper-excitatory conditions, such as those following stroke, an

increase of intracellular calcium activates CaMKII, which is believed to participate in a cascade that can increase damage resulting from excitotoxic events (see Coultrap et al., 2011). For instance, CaMKII may increase and prolong calcium overload by increasing cation conductance of AMPARs (Liu & Zukin, 2007), L-VGCCs (Grueter et al., 2006), and acid sensing ion channels (Gao et al., 2005); thus, enhancing the overexcited state and potentially exacerbating cell death. However, some downstream targets of CaMKII may promote cell survival including phosphorylation and inhibition of nitric oxide synthase (Osuka et al., 2002), insertion of inhibitory $GABA_A$ receptors (Mardsen et al., 2010), and activation of Ras/ERK (Illario et al., 2003). These opposing actions of CaMKII may be triggered by different sources of calcium, and raise the possibility that the most effective treatment for stroke might be one that inhibits detrimental effects but enhances the neuroprotective effects of CaMKII.

NMDA receptors have been targets of intervention following stroke by inhibitory drugs which block downstream pathway activation, however these interventions have been largely unsuccessful when tested in humans (Villman & Becker, 2007; Lau & Tymianski, 2010). Alternatively, inhibition of CaMKII has been shown to enhance cell survival following high glutamate exposure if inhibition follows the stimulus and is short lasting (Vest et al., 2010); however, CaMKII inhibition results in cell death if inhibition is greater than four hours in duration (Ashpole et al., 2012).

Inflammatory response

The inflammatory response pathway induces release of signaling molecules, termed chemokines, in areas that are affected by traumatic events such as stroke or blunt force

trauma (Smith, 2013). Acute exposure to chemokines is intended to lead pro-inflammatory response cells to the area of trauma, but sustained chemokine exposure to nearby neurons results in cell death (Shepherd et al., 2012). Specifically, prolonged CXCR4 activation by CXCL12 (formerly referred to as SDF-1alpha) has been shown to result in increases of cell death in culture. CXCL12 binds to its receptor CXCR4, which is a G-protein coupled protein. Upon binding of a ligand, CXCR4 triggers release of calcium from intracellular stores and the possible activation of CaMKII (Shakiryanova et al., 2011; McCord et al., 2013). Thus, CXCR4 activation may activate CaMKII following ischemia.

Calcium sources

Calcium influx into the cytosol is a highly regulated process that allows for calcium to act as a reliable signaling molecule responsible for the activation of a multitude of pathways. Extracellular concentrations of calcium are on the order of 10^{-3} mM while intracellular concentrations 10^{-7} mM are several orders of magnitudes lower. While there are many possible modes for intracellular Ca^{2+} to become elevated, three particular mechanisms are of interest to excitotoxicity and chemokine-activity: 1) influx through the NMDA-type glutamate receptor; 2) influx through L-type voltage dependent calcium channels; and 3) release from intracellular stores.

NMDA receptors

The primary excitatory neurotransmitter in the mammalian central nervous system is the amino acid, L-glutamate. Glutamate acts on both ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors. Three distinct types of iGluRs are present in the CNS:

1) N-methyl-D-aspartate (NMDA) receptors; 2) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors; and 3) kainate receptors. Upon activation by glutamate AMPA receptors are primarily K^+ and Na^+ permeable and kinetically characterized by their rapid activation and desensitization (1-10 ms). NMDA receptors require glutamate, glycine, and a partially depolarized environment to dislodge a Mg^{2+} ion block to be activated, and are thus thought of as "coincidence detectors" (Yuste et al., 1999). NMDAR activation and desensitization occur on a 10-100 ms timeline and while active, NMDARs are permeable to cations, most notably Ca^{2+}. Activation kinetics and calcium permeability are dependent on the subunit composition of any given NMDAR. In cortex, NMDARs are heterotetramers described as a dimer-of-dimers, requiring two dimers each with an obligatory glycine-activated GluN1 subunit and a glutamate-activated GluN2 (A-D) subunit.

Influx of Ca^{2+} through NMDARs results in the activation of calcium dependent signaling pathways, some contribute to excitotoxicity and others contribute to neuroprotective mechanisms (Arundine et al., 2003). This contribution is currently believed to depend on the subunit composition of NMDARs—evidence currently indicates that GluN2B-containing NMDARs contribute pro-death signals while GluN2A-containing NMDARs contribute pro-survival signals (Mony et al., 2009). It appears this is a least partially due to the specific characteristics of the GluN2B C-tail. The long 2B-tail allows for significant interactions with CaMKII (Bayer et al., 2006), which may situate CaMKII in a way that over-activates the kinase and sets many cell-death pathways in motion (Martel et al. 2010). Currently, GluN2B-containing NMDARs are the target of allosteric modulators for potential therapeutic benefits related to excitotoxicity (Mony et al.).

As mentioned previously, drugs targeting NMDA receptors have been utilized as an intervention following stroke by, however these interventions have been largely unsuccessful when tested in humans (Villman & Becker, 2007; Lau & Tymianski, 2010). For example, derivatives from known negative allosteric modulators (i.e., ifenprodil), have failed to produce decreases in stroke related cell death in clinical trials (Tahirovic et al., 2008). This lack of effect may in part be due to the fact that additional routes of calcium influx beyond NMDARs continue to activate pro-death calcium-dependent pathways. The next section examines one of these alternative routes of Ca^{2+} influx.

Voltage gated calcium channels

L-type VGCC's are calcium permeable channels that are voltage dependent. Their activation is known to affect secretion of neurohormones and neurotransmitters, gene expression, mRNA stability, synaptic efficacy, and the activity of other ion channels (Lipscombe et al., 2004). More importantly to the current study, they have also been implicated in ischemic-induced neuronal injury and neuronal survival (Stanika et al., 2012).

L-VGCCs activate at voltages that typically result in depolarization of the cell (Lipscombe et al., 2012). Generally, this occurs following the large cation influx that is mediated by NMDARs, however in ischemic conditions, it is possible that contributions from AMPARs and acid sensing ion channels are enough to induce VGCC activation. This calcium influx may then subsequently activate CaMKII (Rose et al., 2009). This will be important to consider when evaluating the model of cell death that is induced by sustained chemokine signaling.

Intracellular calcium stores

In addition to external-to-internal calcium conductance to increase cytoplasmic calcium, neurons can release calcium from their own intracellular stores in the endoplasmic reticulum (ER). One mechanism that induces the release of intracellular calcium is the activation of inositol triphosphate (IP3) receptors. In chemokine signaling, the activation of chemokine receptors induces activity that creates IP3, which then serves to activate IP3 receptors in the ER (Murdoch and Finn, 2000). Importantly, release of intracellular calcium has been demonstrated to result in activity of CaMKII in some conditions (McCord et al., 2013).

Calcium dependent pathways

CaMKII

CaMKII is a dodecameric serine/threonine protein kinase that is ubiquitously expressed throughout the brain comprising ~2% of total protein (Miller & Kennedy, 1995). Both the α and β form of CaMKII are implicated in regulating many learning and memory processes (see Coultrap et al., 2012), although through different mechanisms (Okamoto et al., 2008). CaMKII activity is triggered by calcium-bound-calmodulin (Ca^{2+}/CaM) following the elevation of intracellular calcium. Ca^{2+}/CaM binding to CaMKII induces a conformational change exposing multiple phosphorylation and substrate binding sites; this conformational change also allows the subunit to bind ATP and phosphorylate substrates (Figure 1; Meyer et al., 1992). CaMKII can autophosphorylate at multiple sites in its regulatory domain, including threonine 286 (T286); T286 phosphorylation occurs between the autoinhibitory and

substrate binding sites, which keeps the subunit locked open and active if intracellular Ca^{2+} levels drop and CaM dissociates (termed autonomy) (Colbran et al., 1989; Bayer et al., 2001). CaMKII translocation to synapses is enhanced following T286 phosphorylation (Strack et al., 1997) however, it is not necessary (Shen & Meyer, 1999). Synaptic translocation of CaMKII allows it to binds and/or phosphorylate numerous substrates that are involved in learning and memory, as well as ischemia-induced cell death (Coultrap et al, 2011; 2012). Once $Ca^{2+/}$CaM has dissociated from the CaM binding site, T305/306 "burst" autophosphorylation can occur; inhibiting further $Ca^{2+/}$CaM binding and rendering the kinase inactive (Colbran & Solderling, 1990). In this state, CaMKII can no longer phosphorylate any proteins, and thus may help decrease any activation of CaMKII-dependent cell-death pathways.

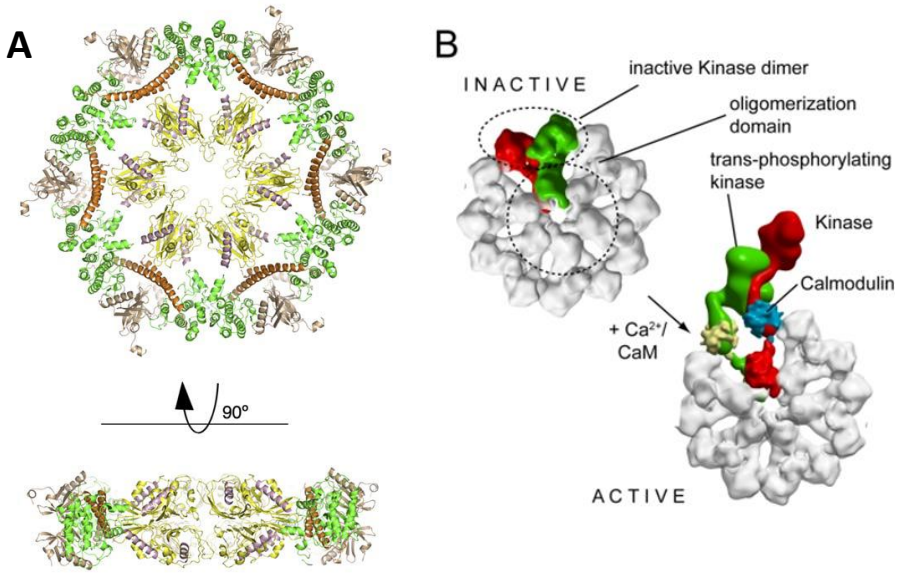

Figure 1. Cartoon depiction of CaMKII. A. Ribbon structure of CaMKII dodecamer. The entire enzyme is around 120Å wide and 60Å thick (Ribbon animation; Rosenberg et al., 2005). B. Bubble structure of inactivated and activated kinase demonstrating the conformational change induced by Ca^{2+}/CaM exposing the phosphorylation sites. Each CaMKII subunit consists of three domains: the C-terminal association domain, which binds to neighboring subunits; the regulatory domain, which includes the majority of the known phosphorylation sites, as well as the CaM binding autoinhibitory domain; and the ATP binding N-terminal catalytic domain (Rosenberg et al., 2005; Bubble structure from Rellos et al., 2010).

Due to high glutamate levels, CaMKII translocation to the synapse is observed following excitotoxic insults (Vest et al., 2010). As noted earlier, several substrates are targeted by CaMKII following excitotoxic insults, some of which are thought to induce cell death and while others promote survival (Coultrap et al, 2011). One of the primary targets of CaMKII following ischemia is the GluN2B subunit of the NMDA receptor which is thought to be cell-death related; this is due in part to the fact that when CaMKII is bound to GluN2B, it will remain in a locked, open state and may phosphorylate cell-death inducing substrates (Bayer et al., 2001; 2006; Barria et al., 2005). Additional death inducing targets include ion channels such as AMPARs (Liu & Zukin, 2007), L-VGCCs (Grueter et al., 2006), and acid sensing ion channels (Gao et al., 2005); targeting of these channels are thought to enhance the overexcited state through influx of cations and exacerbate cell death.

Pro-survival targets include: phosphorylation and inhibition of nitric oxide synthase, which would otherwise induce oxidative stress and eventually induces apoptosis (Osuka et al., 2002); insertion of inhibitory $GABA_A$ receptors, which allows for the influx of anions to counteract the influx of cations (Mardsen et al., 2010); activation of Ras/ERK which serves to counteract apoptosis related phosphorylation (Illario et al., 2003, El Gaamouch et al., 2012); and activation of cAMP response element-binding (CREB), which binds to CRE and increases gene transcription of pro-survival genes (Wheeler et al., 2008).

Due to the number of cell-death causing targets, inhibiting CaMKII activity has been studied as a means to reduce cell death following stroke (Vest et al. 2010; Ashpole et al., 2011). In several studies, inhibition of CaMKII activity following stroke with potent inhibitory peptides has resulted in the survival of neurons in culture, as well as decreased

infarct size in rodent models of stroke (Coultrap et al., 2011; Ashpole & Hudmon, 2011). However, prolonged inhibition of CaMKII lasting >6 hours appears to be detrimental to neurons (Ashpole & Hudmon, 2011). One goal of the current study is further investigation into CaMKII inhibition following chemokine and glutamate induced CaMKII activity.

Inflammation Response

In addition to excitotoxic pathways, inflammatory response pathways become activated following ischemia. Leukocyte influx and activation of microglia are initial phases of the inflammatory response (Becker, 1998). In order for leukocytes and microglia to migrate to the affected area following stroke, they chemotax along high concentration gradients of chemotactic cytokines, or chemokines (Gouwy et al., 2004).

Chemokines

Chemokines are signaling proteins released from neurons and glia that induce chemotaxis from nearby inflammatory response cells. However, they also directly couple neurons through binding of metabotropic chemokine receptors (Meucci et al., 1998). Four distinct types of chemokines exist: C, CC, CXC, and CX_3C, named for the characteristic cysteine residue at the N-terminal region of the chemokine (Fernandez et al., 2002). Here, I will focus on the CXC-type because of their implication in the death of neurons as a result of stroke.

Following stroke, chemokine expression (CXCL12, specifically) is upregulated in the penumbra both by glia and neurons (Hill et al., 2004). This upregulation of CXCL12 is likely intended to bind to CXC-receptors (CXCRs) on microglia as a chemotactic signal to

inflammatory response cells, however CXCRs are also present in neurons (Meucci et al., 1998). Activation of chemokine receptors by chemokine ligands is important during development for helping to control axonal migration; additionally, CXCL12 may act as a neuromodulator in mature cells (Guyon and Nahon, 2007).

Chemokine receptors

In neurons, CXC-receptors are G-protein coupled receptors with 7 transmembrane domains (Fernandex et al., 2002). The specific receptor for CXCL12 is CXCR4. CXCR4 is predominately distributed throughout the somatodendritic region of hippocampal neurons (Shepherd et al., 2012). Upon binding of a ligand, the G-protein creates IP3 which translocates and triggers the release of calcium from intracellular stores (Murdoch and Finn, 2004). This calcium release can then activate CaM and the associated calcium dependent processes previously discussed.

In pathological states, CXCL12 may be both protective and detrimental following activity that mimics stroke (Shepherd et al., 2012). Specifically, CXCR4 activation by CXCL12 has been shown to increases cell death following prolonged activation (Figure 2). In addition, it has been shown that down-regulation of CXCR4 with the anti-inflammatory drug dexamethasone is neuroprotective and reduces infarct size in rodent models of hypoxia/ischemia (Felszeghy et al., 2004). Release of intracellular calcium has been demonstrated to activate CaMKII following oxidative stress (McCord et al., 2013) thus indicating the potential role of CaMKII activation in CXCR4-mediated cell death.

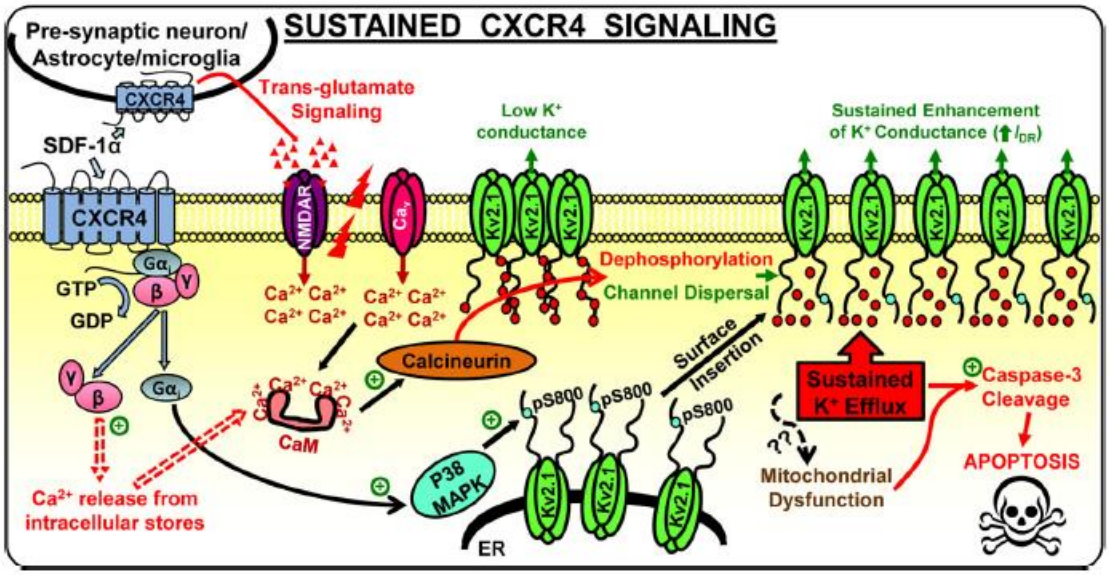

Figure 2. Sustained CXCR4 signaling. CXCL12 is released from cells in the ischemic penumbra, this then binds to CXCR4, which is present in hippocampal neurons; prolonged CXCR4 activation then induces rises in intracellular calcium that combine with NMDA/VGCC mediated calcium rises to activate CaM. Subsequently, CaM activates Calcineurin (a protein phosphatase that dephosphorylates substrates) which activates voltage gated potassium channels subsequently allowing excessive potassium efflux. Following this, cells become stressed, and the apoptosis pathway is triggered. Not shown is the activation of CaMKII (Shepherd et al., 2012).

Proposed model

The proposed model of CXCL12 induced cell death is as follows (from Shepherd et al., 2012):

1) CXCL12 is released from cells in the ischemic penumbra
2) CXCR4 receptors become activated;
3) Prolonged CXCR4 activation (3 hour) induces rises in intracellular calcium that combine with NMDA/VGCC mediated calcium rises to activate CaM.
4) CaM activates Calcineurin (a protein phosphatase that dephosphorylates substrates) which activates voltage gated potassium channels and subsequently allows excessive potassium efflux;
5) Cells undergo mitochondrial dysfunction and the apoptosis pathway is activated.

In contrast to prolonged CXCL12 exposure, acute CXCL12 exposure (30 min) appears to induce a calcium spike which is mitigated by acute outward potassium conductance through voltage gated potassium channels. The present study examined if CXCR4 signaling enhances CaMKII activity that may mediate cell death.

Concentrations of 500 µM glutamate are commonly used as a reliable excitotoxic stimulus when studying glutamate's intracellular effects, while concentrations of 100 µM are used to produce a moderate, excitatory stimulus (Vest et al., 2010; Shen & Meyer, 1999). Here, I examined if these two different concentrations of glutamate (500 µM and 100 µM) interact with the effects of CXCR4 activation (acute and sustained), to produce increased cell

death or attenuated cell death. The data collected suggests that different calcium sources act synergistically to increase a detrimental calcium signal.

Specific Aims

This thesis research utilized cultured hippocampal neurons to investigate the role of CaMKII in CXCL12-dependent cell death. In order to determine the role of CaMKII in the cellular response to CXCL12, immunocytochemistry was used to determine the localization of CaMKII following CXCL12 exposure and subsequent glutamate + glycine exposure. Specific aims were as follows:

1. Establish cell death ratios following acute and sustained CXCL12 exposure and a subsequent glutamate + glycine challenge.
2. Establish CaMKII localization patterns following CXCL12 and a subsequent glutamate + glycine challenge.
3. Inhibit CaMKII activity during CXCL12 pre-exposure and examine if rates of cell death change following a subsequent glutamate + glycine challenge.
4. Inhibit CaMKII activity during CXCL12 pre-exposure and examine if CaMKII localization patterns change following a subsequent glutamate + glycine challenge.

Chapter 2: Methods

Ethical considerations

All experiments involving the use of rats and the procedures were approved by the Western Washington University Institutional Animal Care and Use Committee and were in strict accordance with the *Guide for the Care and Use of Laboratory Animals* described by the National Institutes of Health.

Primary culture of rat hippocampal neurons

Hippocampal neurons from rat embryos were isolated and cultured as described by Kaech and Banker (2006). Hippocampi from Sprague-Dawley rat embryos of either sex were removed at embryonic day 18 and incubated in Hank's Buffered Saline Solution (HBSS; Gibco) with trypsin (1 mg/ml; Gibco) for 15 min at 37°C. Hippocampi were then washed six times with HBSS, followed by trituration to dissociate cells. Cells were then counted and plated onto poly-D-lysine-coated (MW > 100,000; Sigma) glass coverslips at a density of 150,000 cells per 60 mm dish in Neurobasal medium containing (Gibco) B27 (Gibco), 0.6 mM glutamine and penicillin/streptomycin. The cells were incubated in a 5% CO2 incubator at 37°C for 2–4 h, after which the coverslips were transferred to a previously prepared dish containing a glial feeder layer (as described by Kaech and Banker) in Neurobasal/B27/glutamine media and maintained at 37°C in a 5% CO2 incubator. One-third

of the medium was exchanged weekly and APV was added twice weekly. All experiments were performed on neurons that were cultured for 14–16 days *in vitro* (DIV).

Experimental treatment

Coverslips were removed from their glial dishes and briefly washed in extracellular solution (ECS; in mM: 168 NaCl, 2.4 KCl, 10 HEPES, 10 D-glucose, 1.3 $CaCl_2$, 1.3 $MgCl_2$, pH 7.3) and placed face up. 100µL of 100nM CXCL12 (Peprotech; and produced by Dr. John Antos, WWU Chemistry Department) in ECS was applied to each coverslip for either 30 minutes (acute treatment) or 3 hours (sustained treatment). Subsequently, 500µM or 100µM glutamate + 10 µM glycine in ECS was applied for 1 min. The glutamate + glycine solution was then removed and replaced with ECS and incubated for an additional 15 min or 60 min. Cells were treated with a cell death assay and immunocytochemistry (ICC) at the end of their prescribed incubation time.

To assess the role of CaMKII, the CaMKII/CaM inhibitor peptide SC3037 (Santa Cruz Biotechnology; Sequence: LKKFNARRKLKGAILTTMLA) and the CaMKII inhibitor SC3039 (Santa Cruz Biotechnology; Sequence: MHRQEAVDCLKKFNARRKLKGA) were used (final concentration: 1 µM) to inhibit the activity of CaMKII during the CXCL12 incubation and glutamate + glycine stimulations. Peptides inhibitors were co-administered with CXCL12 and remained until washout of glutamate + glycine. See Appendix D for model depicting action of SC3037 and SC3039.

For controls, neurons were incubated in ECS for the corresponding duration of the experiment and were then treated with the same cell death assay followed by the ICC protocol.

Cell death assay and immunocytochemistry

Following experimental treatment, neurons were incubated for 15 min at room temperature (RT) in fixable viability dye eFluor-660 (eBioscience) as a marker of compromised cells destined to dye, immediately followed by 12 min RT incubation in 37°C 4% paraformaldehyde to permanently fix cells. Cells were then permeablized in 0.25% Triton X, and blocked in 10% bovine serum albumin for 30 min at 37°C. Primary antibodies mouse monoclonal anti-CaMKII IgG1 (1:1000, Abcam) and mouse monoclonal anti-PSD95 IgG2a (1:1000, Abcam), were applied at RT overnight on a shaker. Secondary antibodies Alexa Flour 568-conjugated anti-mouse IgG1 (1:800) and Alexa Flour 488-conjugated anti-mouse IgG2a (1:400) were applied for 45 min at 37°C. Cells were mounted on glass slides in DAPI mounting medium (Southern Biotechnology) and sealed with nail polish. Slides were imaged using an Olympus IX-80 inverted microscope with epifluorescence. All images were collected with a CCD camera (Hamamatsu) using Metamorph basic software (Molecular Devices, Inc.). See Appendix A and B for sample images.

Data analysis

To assess cell death, all groups were imaged with a 20X objective and three channels were collected; DIC, CY5 (to image the cell death marker), and DAPI (to image nucleus-

bound DAPI). Each image yielded 10-60 neurons; each coverslip was imaged in 5-10 different locations. To analyze each image, DAPI stained cells were counted to get the total cell count and CY5 stained cells were counted to get the dead cell count. Cell death ratios were calculated. $Cell\ Death\ \% = \frac{CY5}{DAPI}$

To assess CaMKII localization, all images were collected with a 40X oil objective and three channels were collected; DIC, FITC (to image PSD95), Texas Red (to image CaMKII). Five to ten neuron images per coverslip were collected. To quantify CaMKII co-localization with PSD95, the ImageJ (NIH) plug-in JaCOP was used to measure and compare the degree of overlap between CaMKII puncta and PSD95 puncta.

Data were analyzed using SPSS (IBM) with a one-way ANOVA to compare means within each group. Dunnett's post-hoc test was used to compare means in each cell death condition to the CXCR4 condition. REGW-Q post-hoc test was used to compare all CaMKII conditions to each other.

Chapter 3: Results

Glutamate + glycine results in cell death

Exposure to glutamate + glycine significantly increased cell death F (4, 62) = 14.81, p < .0001. REGW-Q post-hoc was used to compare all means and keep a family-wise alpha of .05. REGW-Q post-hoc comparisons revealed that ECS, and the two 100µM were not significantly greater than ECS. However, 15 min post 500µM glutamate + glycine showed significant increases in cell death, while 60 mins post 500µM glutamate + glycine showed significantly more cell death than at 15 min post 500µM glutamate + glycine (Figure 3).

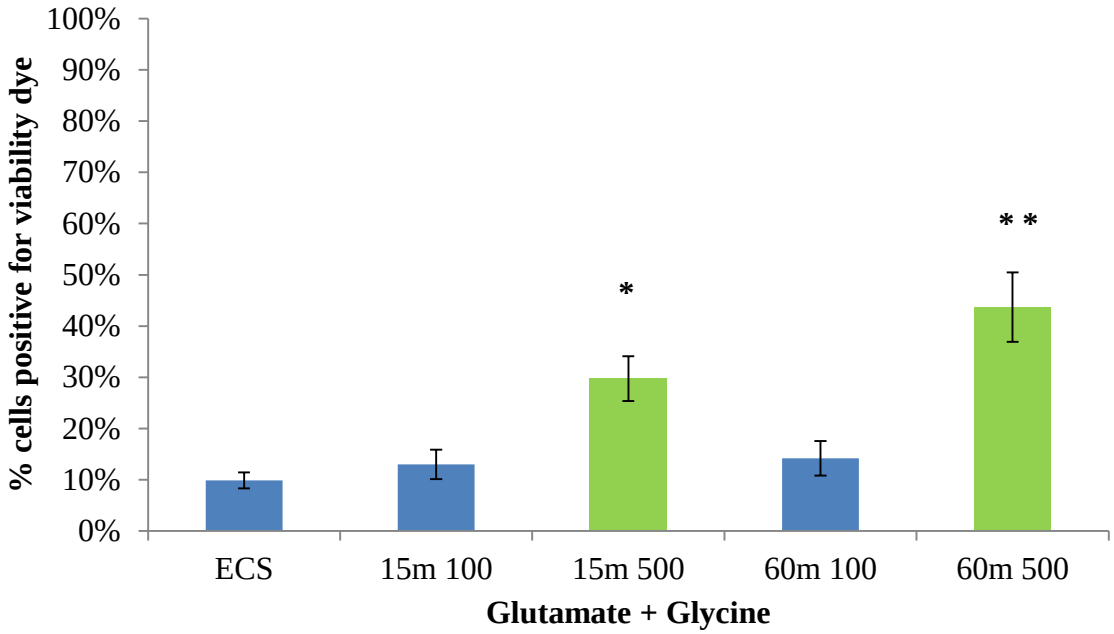

Figure 3. Glutamate + Glycine viability dye ratios. * indicates significant comparison to ECS, ** indicates significant comparison to 500µM glutamate + glycine, $p < .05$ for all comparisons.

CXCL12 enhances glutamate induced cell death

A one-way ANOVA revealed a main effect of acute CXCL12 exposure and glutamate + glycine on cell death, F (5, 63) = 22.04, $p < .0001$. Dunnett's post-hoc test comparing experimental treatments to ECS control revealed that acute CXCL12 exposure had no effect on cell death ($p > .05$). However, cell death levels following 100µM and 500µM glutamate + glycine visualized 15 mins and 60 mins post-treatment were significantly higher than controls ($p < .05$) (Figure 3a).

A one-way ANOVA revealed a main effect of sustained CXCL12 exposure and glutamate + glycine on cell death, F (5, 75) = 103.608, $p < .001$. Dunnett's post-hoc test comparing experimental treatments to ECS control revealed that acute CXCL12 exposure had a significant increase on cell death ($p < .05$). Interestingly, cell death levels following 100µM and 500µM glutamate + glycine visualized 15 min- and 60 mins post-treatment were significantly higher than glutamate + glycine controls ($p < .05$) (Figure 3b).

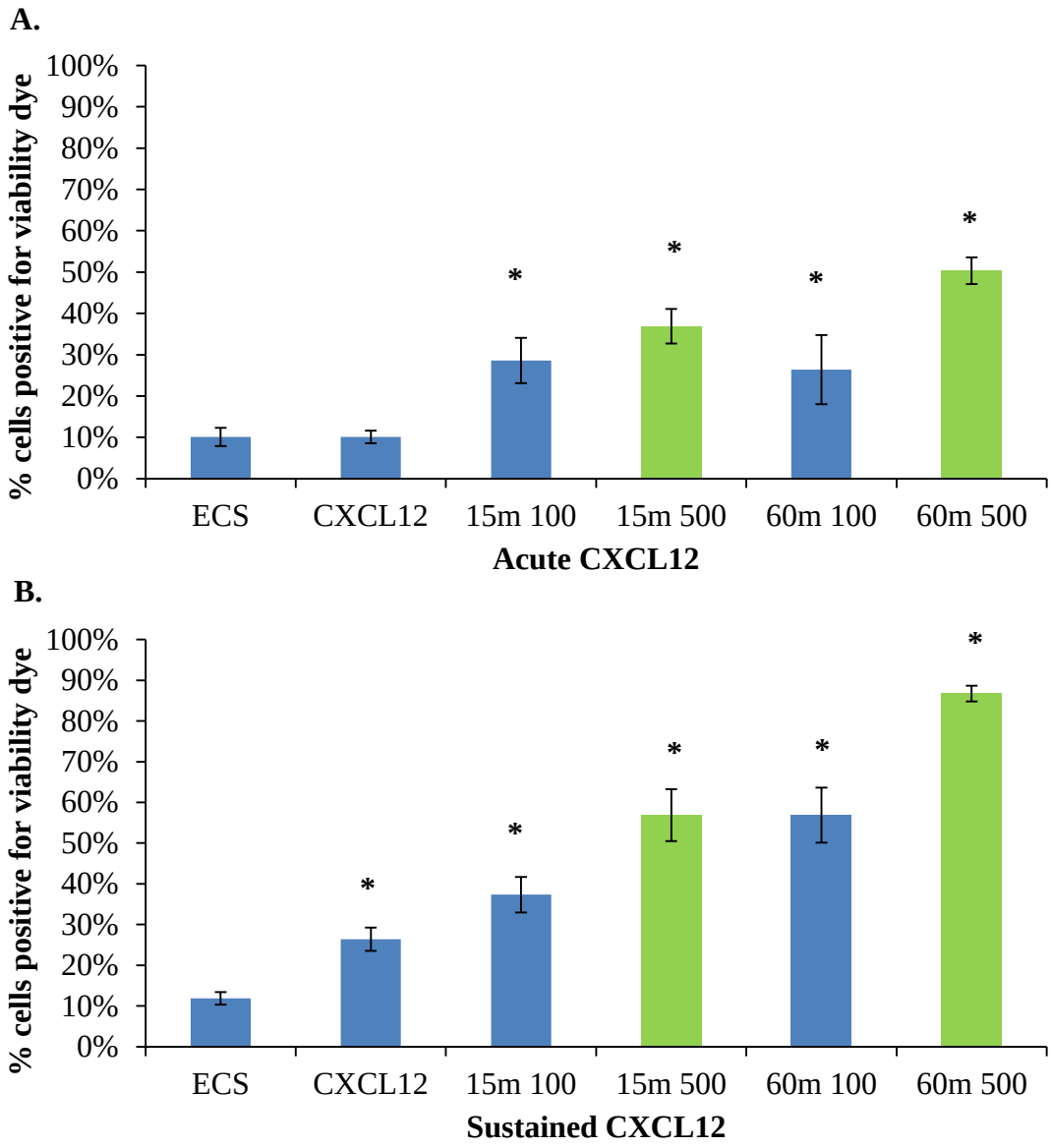

Figure 4. CXCL12 viability dye ratios. * indicates significant Dunnett's post-hoc comparison with ECS as control; $p < .05$ for all comparisons.

SC3037 attenuates glutamate induced cell death following CXCL12 exposure

A one-way ANOVA revealed a main effect of acute CXCL12 exposure combined with the CaMKII inhibitor SC3037 and glutamate + glycine on cell death, $F(5, 51) = 4.256$, $p = .002$. Dunnett's post-hoc test comparing all treatments to CXCL12 + SC3037 revealed that acute exposure of the chemokine with inhibitor had no effect on cell death compared to ECS ($p > .05$). Additionally, cell death levels following 100µM and 500µM glutamate + glycine visualized 15 min-post treatment and 100µM 60 mins post-treatment were not higher than ECS controls. Interestingly, 500µM glutamate + glycine exposure visualized 60 mins following acute CXCL12 + SC3037 resulted in significantly higher cell death ($p < .05$) (Figure 4a).

A one-way ANOVA revealed a main effect of sustained CXCL12 + SC3037 exposure and glutamate + glycine on cell death, $F (5, 44) = 4.523$, $p < .003$. Dunnett's post-hoc test comparing all treatments to CXCL12 + SC3037 revealed that sustained CXCL12 + SC3037 exposure had a significant increase on cell death compared to ECS controls ($p < .05$). However, cell death levels following 100µM and 500µM glutamate + glycine visualized 15 min-post treatment and at 60 mins post-treatment were not significantly different than CXCR4 + SC3037 ($p > .05$) (Figure 4b).

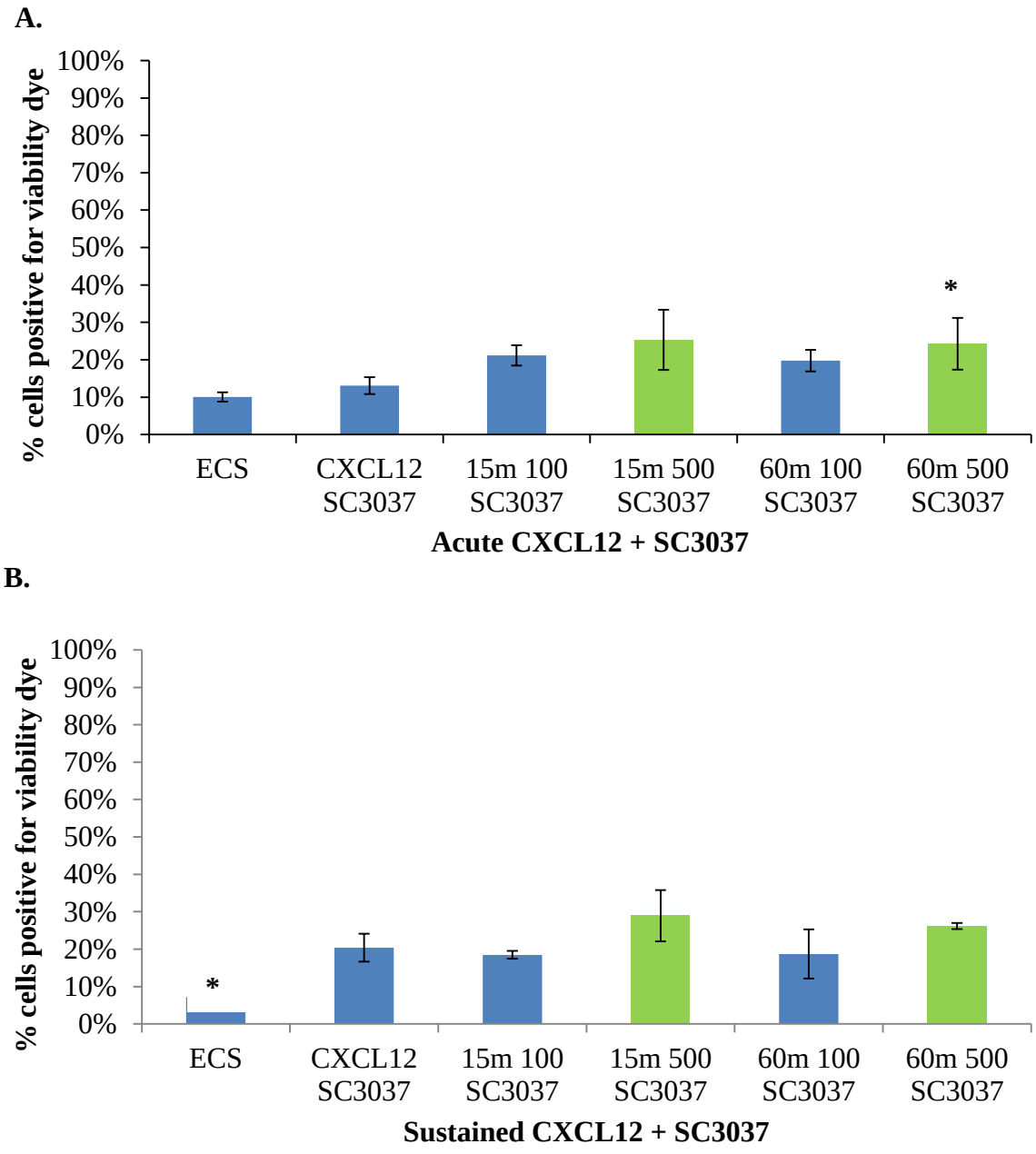

Figure 5. CXCL12 + SC3037 viability dye ratios. * indicates significant Dunnett's post-hoc comparison with CXCL12 + SC3037 as control, $p < .05$ for all comparisons.

SC3039 interferes with CXCL12 induced cell death

A one-way ANOVA revealed a main effect of acute CXCL12 exposure combined with the CaMKII inhibitor SC3039 and glutamate + glycine on cell death F (5, 84) = 4.284, p = .002. Dunnett's post-hoc test comparing all treatments to CXCL12 + SC3039 revealed that acute exposure had no effect on cell death compared to ECS (p > .05). Additionally, cell death levels following 100µM and 500µM glutamate + glycine visualized 15 min-post treatment and 100µM 60 mins post-treatment were not higher than ECS controls. 500µM glutamate + glycine exposure visualized 60 mins following acute CXCL12 + SC3039 resulted in significantly higher cell death (p < .05) (Figure 5a).

A one-way ANOVA revealed a main effect of sustained CXCL12 + SC3037 exposure and glutamate + glycine on cell death F (5, 84) = 7.906, p < .0001. Dunnett's post-hoc test comparing all treatments to CXCL12 + SC3039 revealed that sustained CXCL12 + SC3039 exposure had a significant effect on cell death compared to control (p < .05). Additionally, cell death levels following 100µM and 500µM glutamate + glycine visualized 15 min-post treatment and at 60 mins in the 100µM post-treatment were not significantly different than CXCR4 + SC3039 (p > .05). However, 500µM glutamate + glycine exposure visualized 60 mins following acute CXCL12 + SC3039 resulted in significantly higher cell death (p < .05) (Figure 5b).

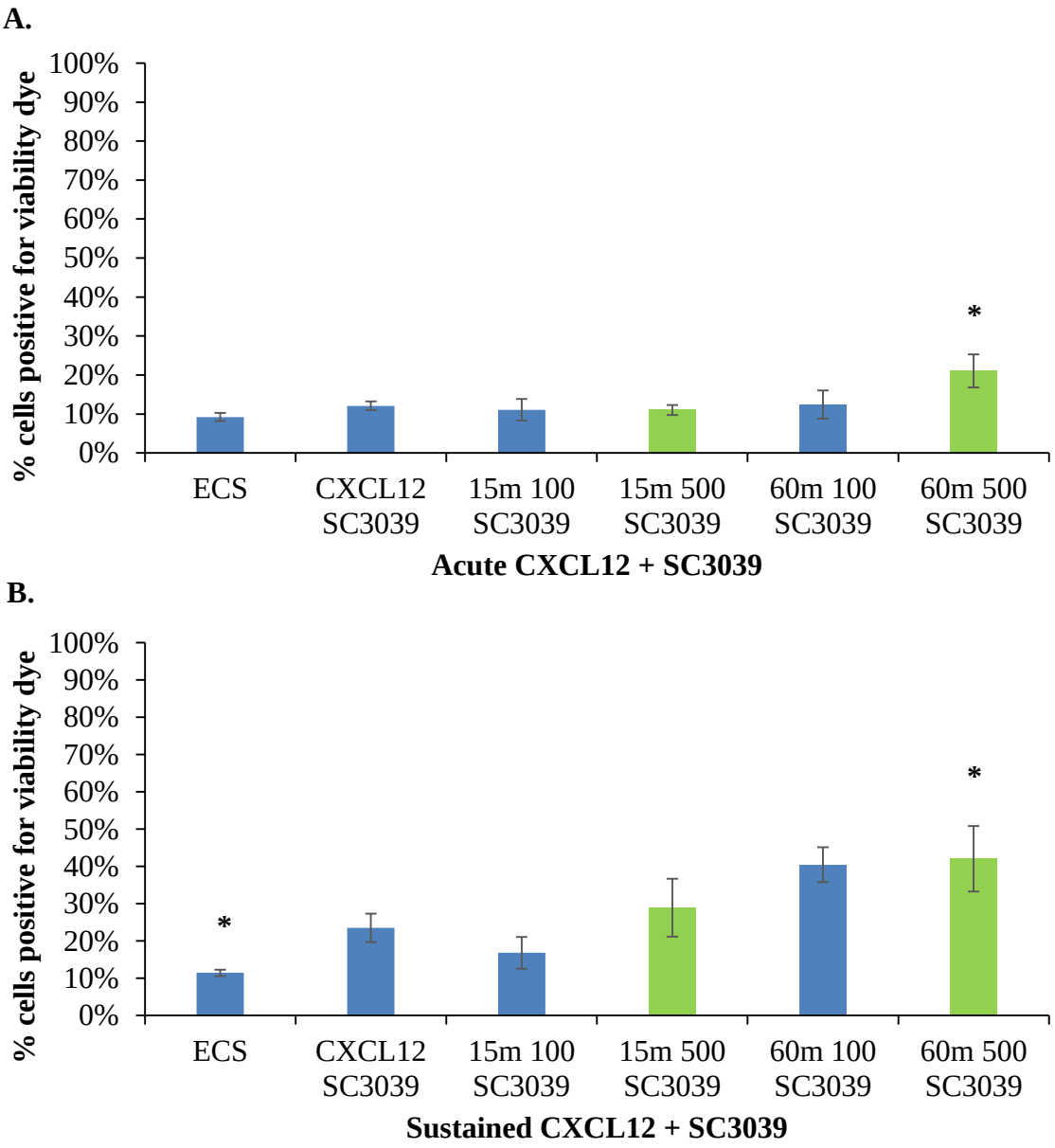

Figure 6. CXCL12 + SC3039 cell death ratios. * indicates significant Dunnett's post-hoc comparison with CXCL12 + SC3039 as control, $p < .05$ for all comparisons.

CaMKII synaptic translocation is induced by CXCL12 and glutamate

A one-way ANOVA revealed a main effect of acute CXCL12 exposure and glutamate + glycine on synaptic CaMKII translocation, F (5, 43) = 10.368, $p < .001$. REGW-Q post-hoc was used to compare all means and keep a family-wise alpha of .05. REGW-Q revealed that all exposure conditions showed significantly greater synaptic CaMKII than ECS as measured by degree of colocalization with the synaptic marker PSD (Figure 7a).

A one-way ANOVA revealed a main effect of sustained CXCL12 exposure and glutamate + glycine on synaptic CaMKII translocation, F (5, 39) = 4.039, $p < .001$. REGW-Q revealed that all exposure conditions showed significantly greater synaptic CaMKII than ECS (Figure 7b).

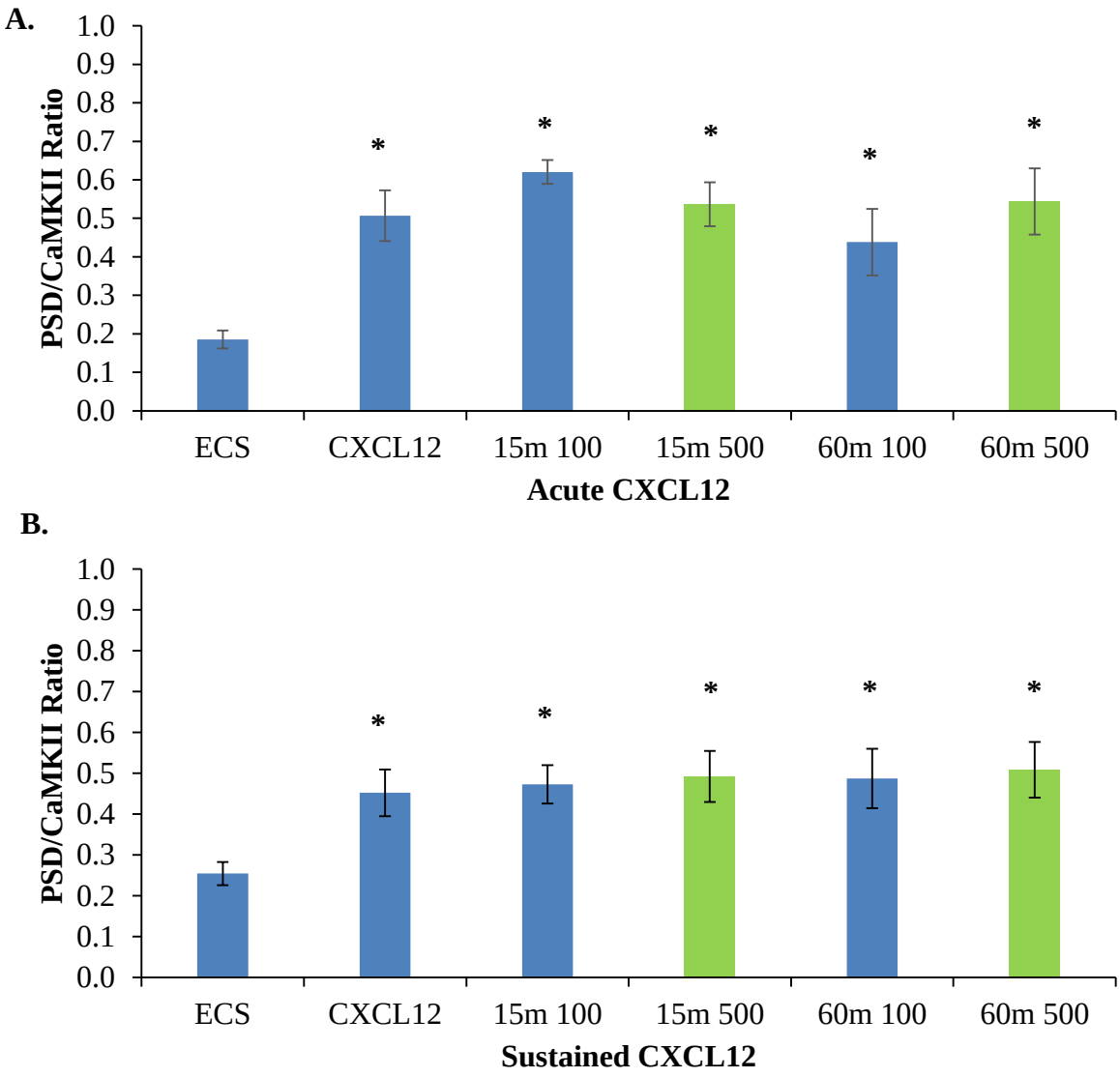

Figure 7. CXCL12 PSD/CaMKIIsynaptic ratios. * indicates significant Dunnett's post-hoc comparison with ECS as control; $p < .05$ for of all comparisons with ECS as control; $p < .05$ for all comparisons.

SC3037 inhibits CaMKII synaptic translocation

A one-way ANOVA revealed no main effect of acute CXCL12 exposure combined with the CaMKII inhibitor SC3037 and glutamate + glycine on CaMKII synaptic translocation F (5, 74) = 1.211, p = 1.211 (Figure 8a).

A one-way ANOVA revealed no main effect of sustained CXCL12 + SC3037 exposure and glutamate + glycine on synaptic CaMKII translocation F (5, 64) = 1.685, p = .151 (Figure 8b).

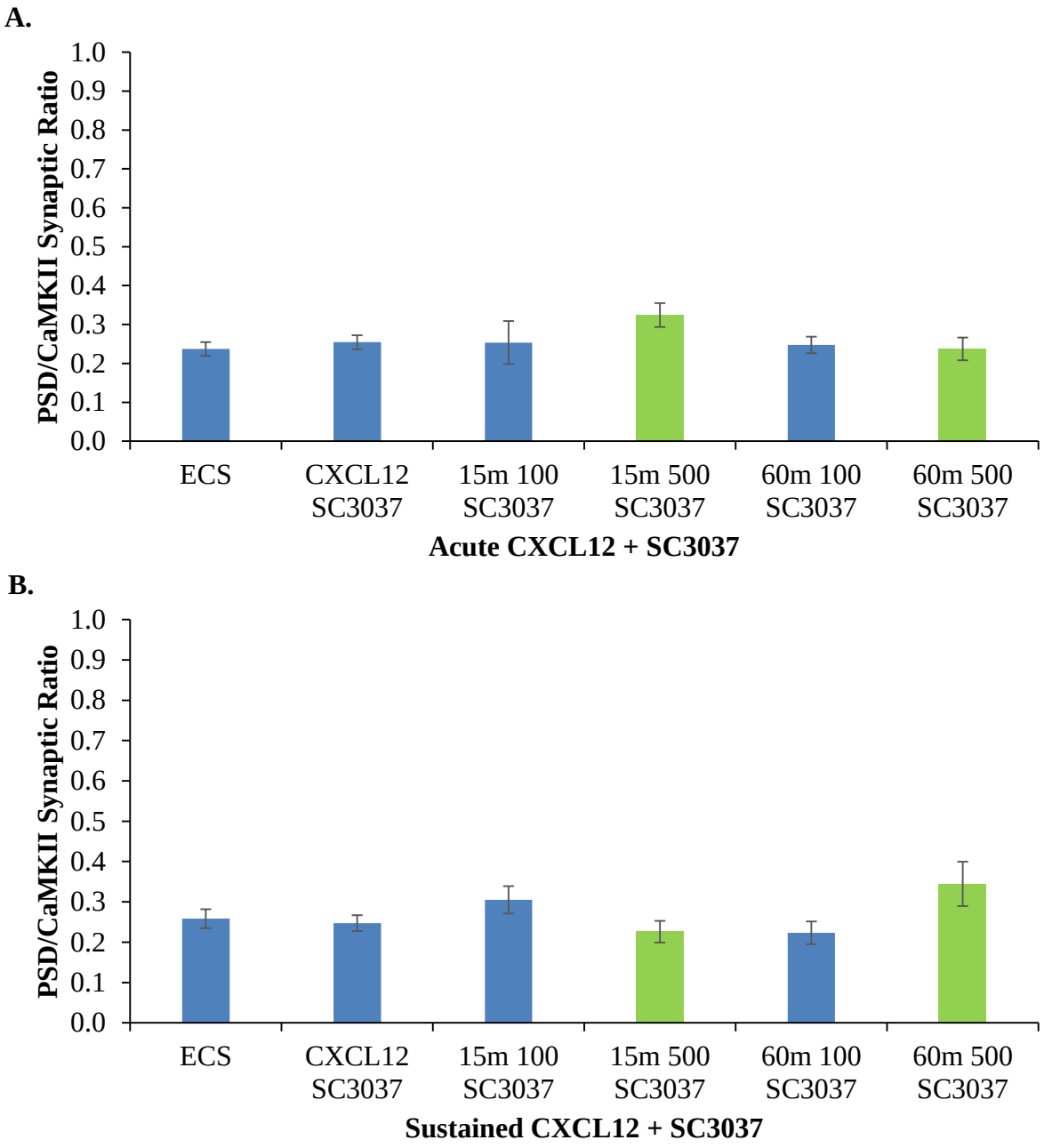

Figure 8. CXCL12 + SC3037 PSD/CaMKII synaptic ratios.

SC3039 allows CaMKII synaptic translocation

A one-way ANOVA revealed a main effect of acute CXCL12 exposure combined with the CaMKII inhibitor SC3039 and glutamate + glycine on synaptic CaMKII, F (5, 101) = 5.47, $p < .001$. REGW-Q revealed that acute CXCL12 exposure and visualization 15 min post- glutamate + glycine had significantly increased PSD/CaMKII ratios compared to ECS, while the 60 min conditions did not (Figure 9a).

A one-way ANOVA revealed a main effect of sustained CXCL12 + SC3039 exposure and glutamate + glycine on cell death F (5, 96) = 4.73, $p = .001$. REGW-Q revealed that sustained CXCL12 + SC3039 exposure and visualization 15 min post- glutamate + glycine had significantly increased PSD/CaMKII ratios compared to ECS, while the 60 min conditions were significantly lower than CXCL12 + SC3039 (Figure 9b).

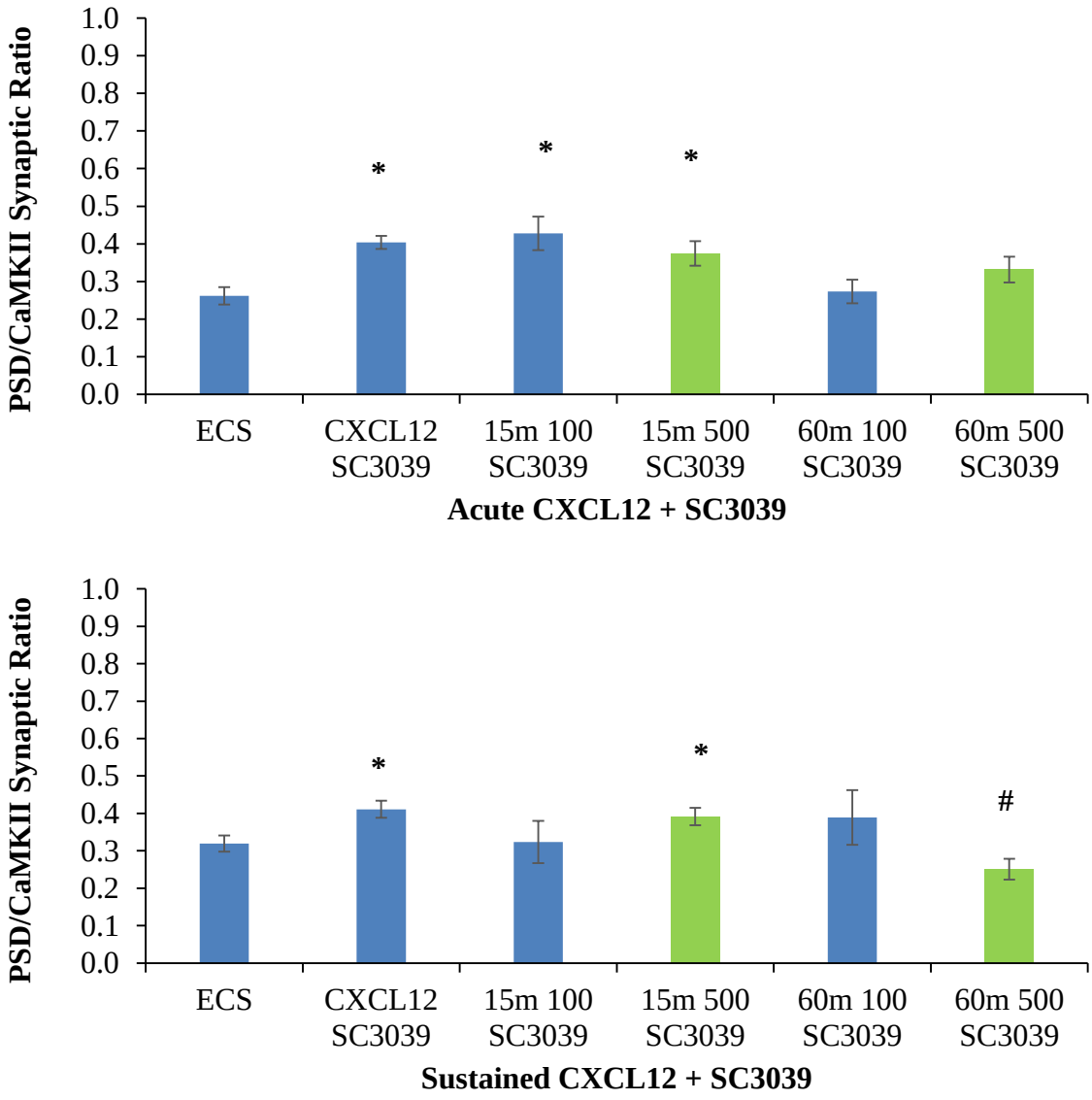

Figure 9. CXCL12 + SC3039 PSD/CaMKII synaptic ratios. * indicates significant difference from ECS; # indicates significant difference from CXCL12; $p < .05$. REGW-Q was used for post-hoc comparisons.

Chapter 4: Discussion

Acute CXCL12 exposure and the corresponding activation of CXCR4 was not harmful to cells, as indicated by uptake of cell viability dye, while sustained exposure resulted in cell death rate of approximately 25% (Figure 4a). These results agree with previous observations of cell death rates following CXCL12 exposure of different time durations (Shepherd et al., 2012). Additionally, both acute and sustained CXCL12 exposure induced synaptic translocation of CaMKII, suggesting CXCL12 induced a rise of intracellular calcium sufficient enough to induce CaMKII translocation (Figure 7a).

When cells were exposed to an excitotoxic stimulus (500µM glutamate + 10µM glycine) following acute CXCL12 exposure, cell death rates rose significantly (Figure 4a). When cells were exposed to a lower, physiologically relevant glutamate stimulus (100µM glutamate + 10µM glycine), cell death rates were similar to those seen following the high concentration excitotoxic stimulus (Figure 4a). Cell death was not observed following a brief sub-maximal glutamate stimulus without CXCL12 pre-exposure (100µM glutamate + 10µM glycine; Figure 3), suggesting that CXCL12 exposure may interfere with the cells ability to buffer against a large calcium influx that results from glutamate + glycine activation of NMDA receptors. Interestingly, following the glutamate + glycine exposure, rates of cell death seemed to plateau by 15 minutes, as neurons examined for cell death at 60 minutes did not show marked increases above the 15 minute levels (Figure 3). This may suggest the cell

death caused by this mechanism occurs rapidly in those cells that die, while cells that survived were still able to buffer against the calcium spike (Ankarcrona et al., 1995).

When cells were challenged with glutamate + glycine following sustained CXCL12 exposure, cell death rates were again higher than expected from both excitotoxic and physiologically relevant concentrations (Figure 4b). However, cell death rates did not plateau after 15 minutes as they did following the acute condition, instead the cell death rate was increased at 60 minutes, suggesting that the brief stimulus may activate slower apoptotic cell death mechanisms. Nicolai et al. (2010) previously demonstrated that prolonged CXCL12 exposure downregulates NMDA receptors and is neuroprotective when cells were subsequently challenged to a sustained 100 μM NMDA stimulus 24 hours later. However, Nicolai et al. were investigating the role of a lower, more physiologically typical level of CXCL12 exposure (20nM CXCL12 compared to 100nM CXCL12 used here) that could be expected in non-ischemic conditions. In this case, CXCL12 down regulates NMDAR gene expression, and in order to see protective effects due to decreased NMDARs, the NMDA stimulus was not administered until 24 hours following initial CXCL12 exposure (Nicolai et al., 2010). It is possible that the CXCL12 paradigm used in the current study also enhances NMDAR downregulation, however it may not occur early enough to protect from a large glutamate challenge within 3 hours.

The CaMKII-derived peptide inhibitor of CaM, SC3037, was able to completely inhibit synaptic translocation of CaMKII following both acute and sustained CXCL12 exposure, as well as following a subsequent application of glutamate (Figure 8). This corresponded to inhibition of cell death following acute CXCL12 exposure and the lower,

physiologically relevant glutamate stimulus (Figure 5a). However, the rate of cell death observed 60 minutes after the application of the excitotoxic stimulus (500μM) was greater than CXCR4 activation alone ($p < .05$). This suggests that inhibition of CaM decreases the CXCL12-induced enhancement of glutamate-induced cell death; however, CaM acts on a multitude of substrates and the inhibition of CaMKII translocation is likely only one of the many effects that CaM inhibition induced.

Inhibition of CaM during sustained CXCL12 exposure did not completely inhibit CXCL12-induced cell death, although it appears to be partially attenuated compared to CXCL12 exposure alone (Figure 5b). This is in agreement with Shepherd et al. (2012), who saw a significant reduction in cell death (but not a return to baseline) through the inhibition of calcineurin, a Ca^{2+}/CaM dependent phosphatase (Figure 2). It may be that the slight reduction in cell death seen here was due to a reduction in calcineurin activity. Additionally, inhibition of CaM reduced cell death observed following additional glutamate stimulus. Together, these data suggest that inhibition of CaM is not sufficient to completely inhibit CXCL12-induced cell death, although it does protect from cell death induced by subsequent glutamate signaling.

The inhibition of CaMKII catalytic activity with the CaMKII-derived peptide inhibitor of CaMKII, SC3039 greatly reduced the rate of cell death following acute CXCL12 exposure and the subsequent glutamate + glycine challenge, suggesting that the inhibition of CaMKII activity protects from this particular excitotoxic stimulus (Figure 6).

SC3039 did not inhibit translocation of CaMKII to the synapse (Figure 9). This was expected because of the nature in which SC3039 inhibits CaMKII. SC3039 binds to the s-site

and a portion of the t-site of the Ca^{2+}/CaM bound CaMKII, inhibiting the ability for ATP binding and subsequent autophosphorylation and autonomy of CaMKII (Bayer et al., 2001; Appendix D). When acute CXCL12 + SC3039 incubated cells were subsequently treated to toxic (500µM) and sub-toxic (100µM) glutamate levels, CaMKII synaptic translocation persisted and was still present at 15 minutes post-treatment. Interestingly, when visualized 60 minutes post glutamate + glycine application, CaMKII synaptic translocation no longer significantly elevated compared to CXCL12 alone. This may have been due to a weak CaMKII interaction with the GluN2B tail, which would allow CaMKII to dissociate and return to the dendrite (Bayer et al., 2006). Another possible explanation is that SC3039 was degraded by proteolysis during the 60 minute incubation (Otmakov et al., 1997) which subsequently allowed ATP binding and burst T305/306 phosphorylation in the absence of Ca^{2+}/CaM. Burst phosphorylation shuts down CaMKII and induces a return of synaptic CaMKII to baseline (Strack, 1997). In either case, it seems CaMKII synaptic translocation alone is not necessary for CXCL12 enhancement of glutamate + glycine induced cell death.

The pattern of CaMKII synaptic translocation following sustained CXCL12 + SC3039 exposure followed a similar pattern of translocation as the acute condition. Again, synaptic accumulation of CaMKII occurred following CXCL12 + SC3039 and glutamate exposure; however, cell death was notably attenuated compared to CXCL12 + glutamate exposure without the CaMKII inhibitor, as well as excitotoxic 500µM glutamate + glycine exposure without CXCL12 exposure (Figure 3). This further supports that inhibition of CaMKII preferentially inhibits glutamate induced cell death following a prolonged exposure to CXCL12.

The role of CaMKII in post-CXCL12 ischemic cell death may help explain why simple glutamate blocking drugs do not always reduce cell death following ischemic attacks (Lau & Tymianski, 2010). Glutamate antagonists are typically only partial antagonists, however the data here suggest that even low glutamate activity can cause cell death following high CXCL12 exposure that may be present following global ischemia. Indeed, work with potent CaMKII-specific inhibitors, with stronger affinity and better specificity (Vest et al., 2007) than the peptides used here, have been able to significantly reduce glutamate-induced cell death, even when administered after a glutamate insult (Vest et al., 2010; Ashpole et al., 2011).

More recently, McCord et al. (2013) demonstrated that CaMKII inhibition greatly reduces activated microglial (AMG) induced cell death. McCord et al. proposed that this cell death is occurs via a mechanism similar to CXCL12 activated cell death proposed by Shepherd et al. (2012); i.e., AMG activates CaMKII through intracellular calcium release, and subsequently CaMKII facilitates insertion of Kv2.1 channels which promote excessive potassium efflux. It is unclear if the current experiment also inhibited Kv2.1 insertion using peptide inhibitors, however it is likely one of the processes that was inhibited as CaMKII phosphorylation of syntaxin is required for Kv2.1 insertion and the detrimental enhancement of potassium efflux (McCord et al., 2013). In addition, although CaMKII inhibition appeared to reduce cell death following sustained CXCL12 exposure compared to non-inhibited conditions, it was not completely attenuated, suggesting CaMKII phosphorylation is necessary for CXCL12 induced cell death, but not sufficient. Thus, other molecular mechanisms are likely triggered by CXCL12 exposure/CXCR4 activation which contribute

to cell death, but inhibition of CaMKII at least partially protects from some of the negative effects of excitotoxicity.

Several types of CaMKII mutants exist which can help distinguish the substrate interactions necessary for CXCL12/glutamate + glycine induced cell death. For example, CaMKII-I205K specifically inhibits GluN2B interaction, but leaves kinase activity intact (Bayer et al., 2006). This would highlight if the GluN2B interaction is necessary. Additionally, CaMKII-T286A impairs autonomy but leaves kinase ability intact during the calcium stimulus (Coultrap et al., 2012); this would demonstrate if CXCL12 cell death can be initiated by brief activity of CaMKII. Through these experiments, it may be possible to resolve any specific interactions are required for CXCL12 induced cell death.

Taken together, the current study supports further investigation of CaMKII as a potential therapeutic target to decrease cell death following global ischemia. Because CaMKII can act on such a multitude of substrates in the synapse and is important for many normal functions, including learning and memory, determining how to inhibit only the specific interaction involved in excitotoxicity-related cell death may be key to utilizing CaMKII inhibition therapeutically.

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Appendix A

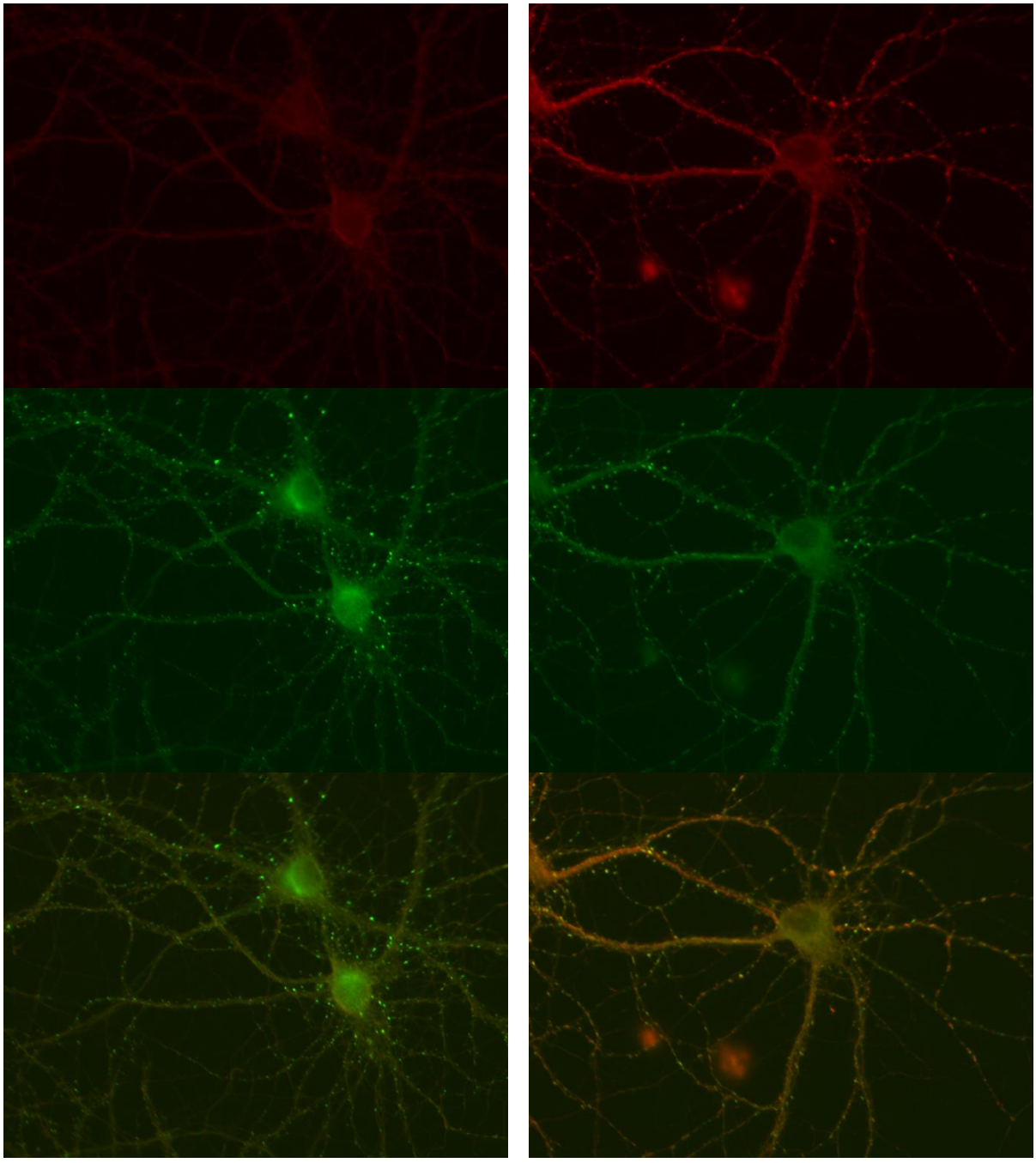

Figure A1. Left: ECS exposed neuron images of CaMKII (red; top), PSD95 (green; middle) and Overlay (multi; bottom). CaMKII appears very diffuse without stimulation of cells. Overlay shows that CaMKII is not colocalized to the synaptic marker protein, PSD95. Right: Acute CXCL12 exposed neuron images of CaMKII (red; top), PSD95 (green; middle) and Overlay (multi; bottom). CaMKII appears punctate. Overlay shows that CaMKII puncta are highly colocalized with PSD95.

Appendix B

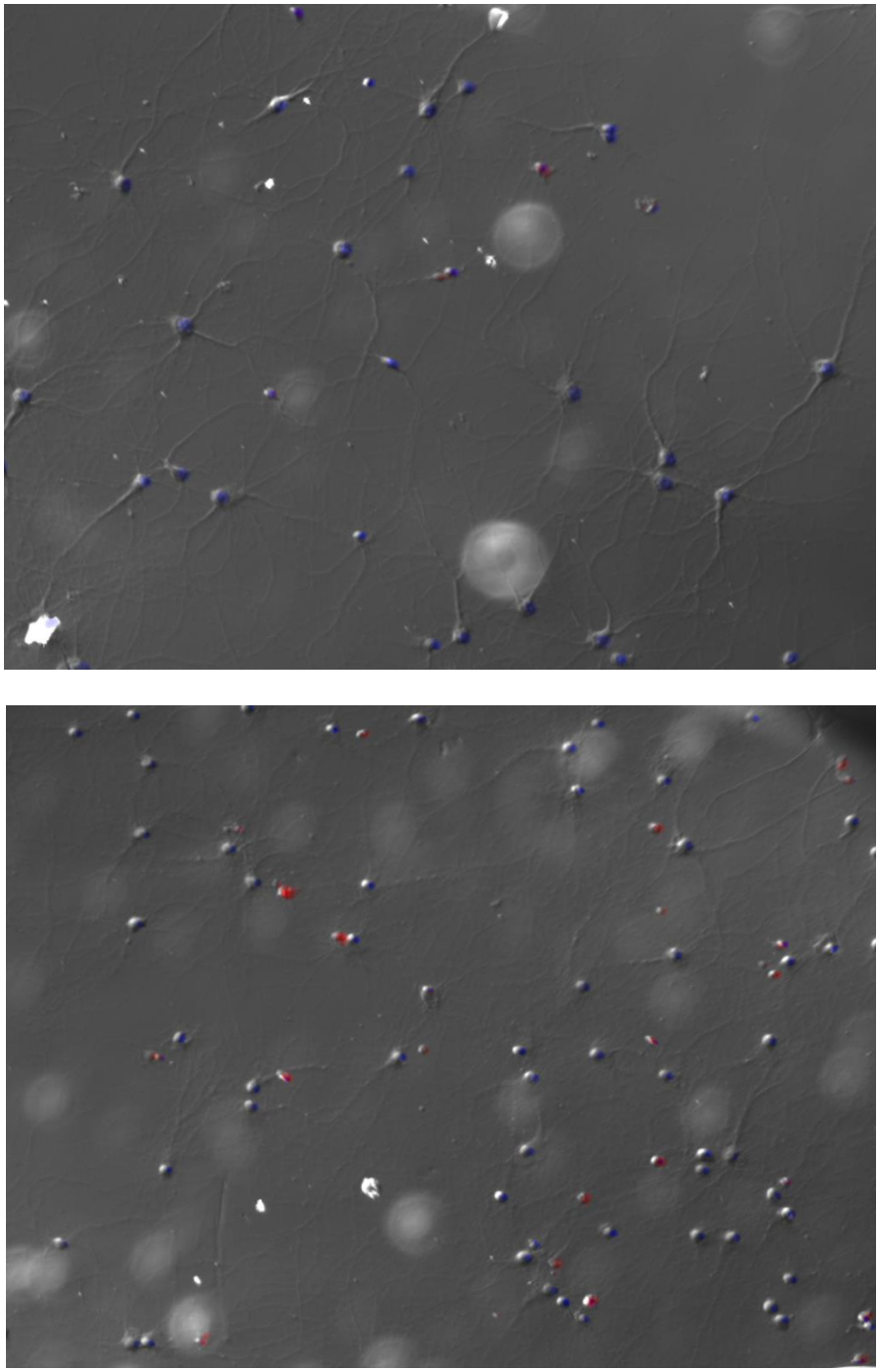

Figure B1. Sample immunohistochemistry neurons. Top: Overlay image of ECS images at 20X includes DIC image with DAPI channel and CY5 channel. Blue indicates all cells, red indicates dead cells. Bottom: Overlay image of acute CXCL12 + Glutamate/Glycine at 15 minutes.

Appendix C

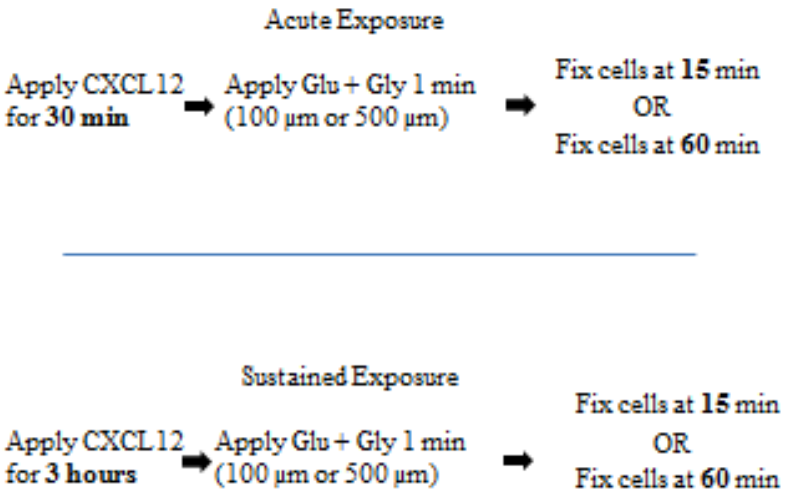

Figure C1. Schematic depicting the order in which CXCL12 and glutamate + glycine are applied. CXCL12 is applied, followed by glutamate + glycine. Cells are then fixed after 15 or 60 minutes. Total experiment time range 46 min to 4:01 hours.

Appendix D

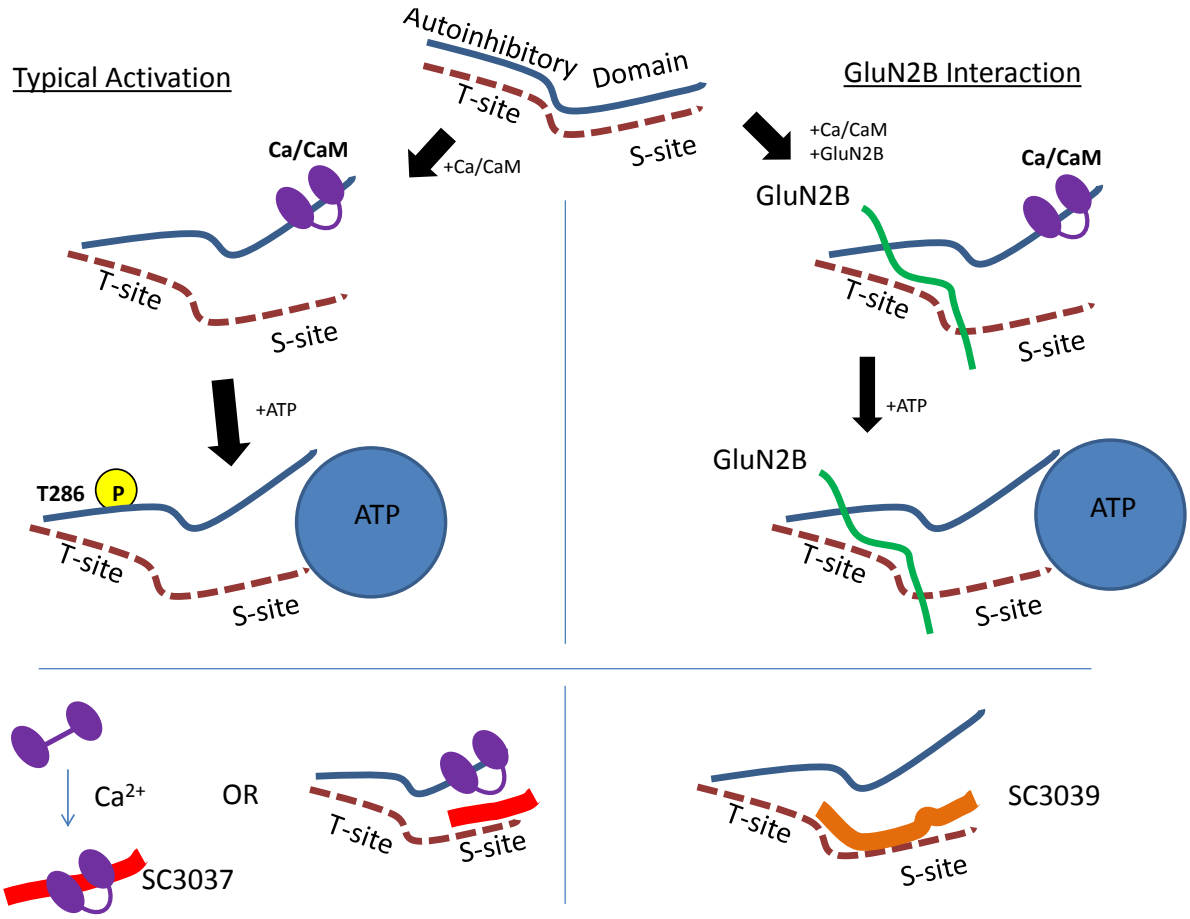

Figure D1. CaMKII regulatory domain is typically autoinhibited by the autoinhibitory domain (blue). When the autoinhibitory domain is bound by Ca/CaM, the t-site and the s-site become exposed. This allows for ATP to bind as well as phosphorylation of T286, both of which can leave the kinase active following the dissociation of CaM (top left). In addition, GluN2B can bind to the t-site of CaMKII to keep the kinase locked in an open position in the absence of CaM (top right). In the presence of SC3039, the t-site only remains partially available for GluN2B interaction, it is unclear if SC3039 allows any GluN2B interaction. However, SC3039 (orange) occupies the ATP binding domain and the kinase is not catalytically active (bottom right). In the presence of SC3037, Ca^{2+}/CaM binds SC3037 when activated; if Ca^{2+}/CaM binds CaMKII, SC3037 can bind CaMKII and inhibit ATP binding. Figure based on data from Bayer et al. (2001; 2006) and Rosenberg et al. (2005).